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Nucleic acids and polypeptides specific for pathogenic strains of the *Neisseria* genus.

The present invention relates to nucleic acids encoding polypeptides specific for pathogenic strains of the *Neisseria* genus, in particular which are useful for preventing or treating a *Neisseria meningitidis* infection.

In general, meningitis is either of viral origin or of bacterial origin. The bacteria mainly responsible are: type b *Haemophilus influenzae*, *Neisseria meningitidis* and *Streptococcus pneumoniae*. The *Neisseria meningitidis* species is subdivided into serogroups according to the nature of the capsular polysaccharides. Although about a dozen serogroups exist, 90% of meningitis cases can be attributed to three serogroups: A, B and C.

Effective vaccines based on capsular polysaccharides exist for preventing meningitis caused by *Neisseria meningitidis* serogroups A and C. These polysaccharides, unmodified, are only slightly immunogenic, or not at all, in children under the age of two, and do not induce any immune memory. However, these drawbacks can be overcome by conjugating these polysaccharides to a carrier protein.

On the other hand, the polysaccharide of *Neisseria meningitidis* serogroup B is non-immunogenic, or relatively non-immunogenic in humans, whether or not it is in a conjugated form. Thus, it appears to be highly desirable to seek a vaccine against meningitis caused by *Neisseria meningitidis*, in particular *Neisseria meningitidis* serogroup B, other than a vaccine based on polysaccharide.

To this end, various proteins of the external membrane of *N. meningitidis* have already been proposed, such as the membrane-bound receptor for human transferrin (WO 90/12591 and WO 93/06861).

*Neisseria meningitidis* is genetically very close to *Neisseria gonorrhoeae* and *Neisseria lactamica*.

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*N. meningitidis*. Only the ORFs with sequences having a maximum frequency of use of these codons were selected. At the end of this analysis, 197 candidate ORFs were selected.

5           The ORFs selected using this double analysis were subjected to a homology search through all of the available banks, using the BLASTX® program, from the access to the Pathoseq® bank of Incyte Pharmaceuticals. This homology search made it possible to exclude the  
10 ORFs encoding, *a priori*, cytoplasmic or periplasmic proteins, in particular metabolism proteins. The ORFs were also subjected to analysis of possible protein motifs, using the DNA Star Protean® program (Lasergene software).

15           The authors of the present invention then investigated whether the ORFs selected at the end of the previous step (118 in number) were effectively absent from *N. lactamica*, as predicted by the application of the prior art WO 98/02547.

20           To this end, a PCR amplification was carried out. This amplification proved to be ineffective for 78 of the 118 ORFs tested. Only the ORFs for which the amplification in *N. lactamica* was negative (sequences named "lactamica<sup>-</sup>") were selected. In order to verify  
25 that these negative results were not "false negatives", the lactamica<sup>-</sup> sequences selected were subjected to a control by dot blot. At the end of this step, only 23 ORFs were confirmed *N. meningitidis*<sup>+</sup>/*N. lactamica*<sup>-</sup>.

30           Finally, these 23 ORFs were repositioned in their entirety on the *N. meningitidis* ATCC13090 genome. This made it possible to demonstrate that three ORFs previously eliminated on the basis of their putative protein function appeared to be located close to, or were even framed by, some of the 23  
35 *N. meningitidis*<sup>+</sup>/*N. lactamica*<sup>-</sup> ORFs. These three ORFs (SEQ ID Nos 29, 35 and 37) were reintroduced into the study, and it was proven that they were also *N. meningitidis*<sup>+</sup>/*N. lactamica*<sup>-</sup>.

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5 The authors of the present invention then attempted to discover whether the ORFs identified using the genome of the *N. meningitidis* serogroup B strain ATCC 13090 were also present in the genomes of  
10 *N. meningitidis* serogroup A Z2491 (Sanger Centre) and of *N. gonorrhoeae* FA1090 (Advanced Centre of Genome Technology, Oklahoma University). Then, they compared the sequences derived from these various genomes, with multiple alignment (Clustal, Infobiogen). This made it  
15 possible to redefine, for some of the ORFs, the most probable position of the initiation codon and translation stop codon. The sequences of the open reading frames derived from the strain ATCC13090 are given in the SEQ ID Nos 1-51 (odd numbers) and the amino acid sequences which are deduced therefrom are given in the SEQ ID Nos 2-52 (even numbers).

20 A subject of the present invention is, therefore, a nucleic acid in isolated form encoding a polypeptide, or an antigenic fragment thereof, excluding the nucleic acids disclosed in SEQ ID Nos 70, 73, 74, 77, 80, 81, 87, 88, 89, 94, 95 and 98 of application WO 98/02547 (sequences attached to the present description and numbered SEQ ID Nos 70A, 73A, 74A, 77A, 80A, 81A, 87A, 88A, 89A, 94A, 95A and 98A so  
25 as to distinguish them from the sequences of the invention); said polypeptide having an amino acid sequence which is identical or homologous to a sequence selected from those of group II; group II consisting of the sequences shown in SEQ ID Nos 2-52 (even numbers)  
30 and the sequence SEQ ID No. 53.

Preferably, said nucleic acid can have a nucleotide sequence selected from those of group I, group I consisting of the sequences shown in SEQ ID Nos 1-51 (odd numbers).

35 The term "nucleic acid" includes and means equally ORF, gene, polynucleotide, DNA and RNA. The term "nucleic acid in isolated form" means a nucleic acid separated from the biological environment in which it is found under natural conditions. For example, a

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DNA molecule exists under natural conditions when it is integrated into a genome or when it forms part of a library of genes. In that case, it cannot be in isolated form. On the other hand, the same molecule separated from the genome by cloning (for example subsequent to a PCR amplification) should be considered as being in isolated form. Typically, a DNA molecule in isolated form does not contain the coding regions which are contiguous with it in 5' and 3' in the genome from which it is derived. The nucleic acids in isolated form can be integrated into vectors (for example plasmids, or viral or bacterial vectors) without, even so, abandoning their characteristic of being separated from their natural environment.

The authors of the present invention have more particularly found that the ORFs which, when they are derived from the strain ATCC 13090, are characterized by the sequences as shown in SEQ ID Nos 19, 27, 39, 45, 47 and 49 are specific for *Neisseria meningitidis* insofar as it has not been possible to demonstrate identical or homologous sequences in the *N. gonorrhoeae* genome. They have also found that the ORF characterized by the strain sequence as shown in SEQ ID No. 39 is specific for *Neisseria meningitidis* serogroup B.

A subject of the invention is also a polypeptide in isolated form, or a fragment thereof; said polypeptide having an amino acid sequence identical or homologous to a sequence selected from those of group II.

The amino acids framed in the sequence SEQ ID No. 8 correspond to the signal sequence, and the amino acid in bold represents the first amino acid of the mature form. The amino acid sequence of the mature protein form is represented in SEQ ID No. 53.

In the context of the present invention, the terms "polypeptide" and "protein" are equivalent and mutually interchangeable. They refer to any amino acid chain, whatever its length and its post-translational

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modifications (for example phosphorylation or glycosylation).

5 The expression "antigenic fragments of the polypeptides specific for pathogenic strains of the *Neisseria* genus" is intended to mean the polypeptides derived from the polypeptides of the invention as defined above, through deletions of portions of said polypeptides without destroying the antigenicity (for example, without notable loss of the antigenic activity) of said polypeptides. The specific antigenicity can be determined using various methods known to those skilled in the art, as explained later.

10 These fragments are preferably at least 12 amino acids long, more preferably at least 20 amino acids long, preferentially 50 amino acids long, more preferably still 75 amino acids long, preferentially 100 amino acids long.

15 These fragments can be used to reveal epitopes which may be masked in the parent polypeptides. They are also advantageous for inducing a T-lymphocyte-dependent protective immune response. The deletions can, in fact, make it possible to eliminate immunodominant regions which are highly variable between various strains.

20 Such fragments can be obtained using standard techniques known to those skilled in the art (for example, Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons Inc, 1994), for example by PCR, RT-PCR or treatment with restriction enzymes for the cloned DNA molecules, or by the method of Kunkel et al. (Proc. Natl. Acad. Sci. USA (1985) 82:448).

25 The expression "homologous amino acid sequence" is intended to mean a sequence which differs from one of the sequences of group II by substitution, deletion and/or insertion of one or more amino acids, at positions such that these modifications do not destroy the specific antigenicity of the polypeptide in question.

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Said substitutions are preferably conservative substitutions, i.e. substitutions of amino acids of the same class, such as substitutions of amino acids with uncharged side chains (for instance asparagine, glutamine, serine, threonine and tyrosine), of amino acids with basic side chains (for instance lysine, arginine and histidine), of amino acids with acid side chains (for instance aspartic acid and glutamic acid) or of amino acids with apolar side chains (for instance glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan and cysteine).

Advantageously, a homologous amino acid sequence has at least a 75% degree of homology (i.e. of identity) with one of the sequences of group II; preferably this degree of homology is at least 80%, most preferably at least 90%. The homologous amino acid sequences include, in particular, the sequences which are substantially identical to one of the sequences of group II. The expression "substantially identical sequence" means a sequence which has at least a 90%, advantageously at least a 95%, preferably at least a 97%, and most preferably at least a 99%, degree of homology (i.e. of identity) with one of the sequences of group II. In addition, it may differ from the reference sequence only through mainly conservative substitutions.

The degree of homology (also named degree of identity) is generally determined using a sequence analysis program (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Centre, 1710 University Avenue, Madison, WI 53705). Similar amino acid sequences are aligned so as to obtain the maximum degree of homology (i.e. identity). To this end, it may be necessary to artificially introduce gaps into the sequence. Once optimal alignment has been produced, the degree of homology (i.e. identity) is established by recording all the positions for which the amino acids

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of the two sequences compared are identical, with respect to the total number of positions.

The expression "homologous nucleotide sequences" is intended to mean sequences which differ from the sequences of group I by substitution of one or more nucleotides, or by deletion and/or insertion of one or more codons, at positions such that these sequences (i) still encode polypeptides having the sequences of group II, due to the effect of the degeneracy of the genetic code; or (ii) encode polypeptides having homologous sequences as defined above.

Advantageously, a homologous nucleotide sequence has at least a 60% degree of homology with one of the sequences of group I; preferably this degree of homology is at least 80%, most preferably at least 90%.

Typically, a homologous nucleotide sequence hybridizes specifically to the sequences complementary to the sequences of group I, under stringent conditions. The temperature at which the hybridization assay is carried out constitutes an important factor which influences the stringency. Conventionally, this temperature, termed hybridization temperature ( $T_h$ ), is selected from 5 to 40°C, preferably from 20 to 25°C, below the temperature at which 50% of the paired strands separate ( $T_m$ ). In general, it is considered that conditions of high stringency are satisfied when  $T_h$  is lower than  $T_m$  by 5 to 25°C approximately, for example by 5 to 10°C or, most commonly, by 20 to 25°C approximately. Moderate stringency is established when  $T_h$  is lower than  $T_m$  by 30 to 40°C.

For sequences comprising more than 30 bases, the temperature  $T_m$  is defined by the equation:  $T_m = 81.5 + 0.41(\%G+C) + 16.6\log(\text{cation concentration}) - 0.63(\%\text{formamide}) - (600/\text{number of bases})$ . Thus, ionic strength has a major impact on the value of  $T_m$ . The temperature  $T_m$  increases by 16.6°C every time the monovalent cation concentration increases by a factor of 10. The addition of formamide into the hybridization

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buffer causes, on the other hand, the value of  $T_m$  to decrease. (For a complete reference, see Sambrook et al., Molecular Cloning, A laboratory manual, Cold Spring Harbor Laboratory Press, 1989, pages 9.54-9.62).

5 Conventionally, hybridization experiments are carried out at a temperature of 60 to 68°C, for example at 65°C. At this temperature, stringent hybridization conditions can, for example, be implemented in 6xSSC, advantageously in 2xSSC or 1xSSC, preferably in  
10 0.5xSSC, 0.3xSSC or 0.1xSSC (in the absence of formamide). A solution of 1xSSC contains 0.15 M of NaCl and 0.015 M of sodium citrate.

For this reason, in other words, a subject of the invention is a polynucleotide in isolated form,  
15 which is capable of hybridizing, under stringent conditions, with a DNA molecule having one of the nucleotide sequences as shown in SEQ ID Nos 1-51 (odd numbers) or the sequences complementary thereto.

A specific class of homologous sequences  
20 consists of those encountered naturally by virtue of the extremely common phenomenon of allelic variation. A bacterial species, for example *N. meningitidis* or *N. gonorrhoeae*, consists of a large variety of strains which differ from one another through minor variations,  
25 termed allelic variations. Thus, a polypeptide which is present in various strains and which, of course, performs the same biological function in each of them, can have an amino acid sequence which is not identical from one strain to the other. In other words, the  
30 sequences derived from the allelic variation are purely sequences equivalent or alternative to those of group II. The class of sequences which are allelic variants of one of the sequences of group II consists of the sequences of the polypeptide as found in a  
35 pathogenic species of the *Neisseria* genus (for example, *N. meningitidis* or *N. gonorrhoeae*) other than the *N. meningitidis* strain ATCC 13090. The biological function which is associated with the allelic variant sequences is the same as that which is associated with

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the reference sequence. The differences (substitution, deletion or addition of one or more amino acids) which they exhibit between one another (including the reference sequence) do not modify the biological function of the polypeptide. The term "biological function" is intended to mean the function exercised by the polypeptide in the cells which produce it naturally.

The allelic variation is also expressed in the coding sequences. A polynucleotide, encoding a polypeptide, having a sequence which is an allelic variant of one of the sequences of group I can be easily cloned by amplifying the genomic DNA of the strains of pathogenic species of the *Neisseria* genus, for example by PCR (polymerase chain reaction), using synthetic oligonucleotide primers capable of hybridizing to the 5' and 3' ends of the coding region. The sequences of such primers can easily be established by those skilled in the art using the nucleotide sequences given in SEQ ID Nos 1-51 (odd numbers). The primers generally have from 10 to 40 nucleotides, preferably from 15 to 25 nucleotides.

For this reason, in other words, a subject of the invention is a DNA molecule in isolated form which can be amplified and/or cloned by PCR from the genome of a pathogenic *Neisseria* strain, using a pair of 5' and 3' PCR primers; the sequences of these primers being established using one of the nucleotide sequences as shown in SEQ ID Nos 1-51 (odd numbers). An example is given, for each pair of primers, in Example I.1 hereinafter.

A subject of the present invention is more particularly the allelic variants having the nucleotide sequences SEQ ID Nos 54 to 76 (even numbers) and the products encoded by these nucleotide sequences, having the amino acid sequences SEQ ID Nos 55 to 77 (odd numbers).

The polypeptides of the invention can be fused to other polypeptides, for example by translation of a

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hybrid gene. Vectors for expressing fusion polypeptides are commercially available, such as the vectors pMal-c2 or pMal-p2 from New England Biolabs, in which the protein to which the polypeptides of the invention can be fused is a maltose-binding protein, the glutathione-S-transferase system from Pharmacia or the His-Tag system from Novagen. Such systems are in particular useful for purifying the polypeptides of the invention. The polypeptides of the invention can be fused to polypeptides having adjuvant activity, such as for example the B subunit of cholera toxin or the B subunit of the *E. coli* heat-sensitive toxin.

The nucleic acids of the present invention can be used (i) in a process for producing the polypeptides encoded by said nucleic acids, in a recombinant host system, (ii) for the construction of vaccination vectors, such as poxviruses, intended to be used in methods and compositions for preventing and/or for treating an infection with pathogenic *Neisseria* strains, in particular with *Neisseria meningitidis*, (iii) as a vaccination agent in a naked form or in combination with a vehicle which promotes transfer to the target cells and, (iv) in the construction of attenuated *Neisseria* strains which can overexpress a nucleic acid of the invention, or express it in a non-toxic, mutated form.

The present invention also provides (i) an expression cassette containing a polynucleotide of the invention placed under the control of elements allowing its expression, in particular under the control of a suitable promoter; (ii) an expression vector containing said expression cassette; (iii) a host cell (prokaryotic or eukaryotic) transformed with an expression cassette and/or an expression vector as defined above, and (iv) a method for obtaining a polypeptide encoded by said polynucleotide of the invention, comprising culturing said transformed cell under conditions allowing the expression of the

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polynucleotide of the invention, and recovering the polypeptide from the cell culture.

Among the eukaryotic hosts which can be used, mention may be made in particular of yeast cells (for example *Saccharomyces cerevisiae* or *Pichia Pastoris*), mammalian cells (for example COS1, NIH3T3 or JEG3) arthropod cells (for example *Spodoptera frugiperda* (SF9)) and plant cells. Among the prokaryotic hosts which can be used, mention may be made in particular of *E. coli*.

The choice of the expression cassette depends on the host system chosen, and also on the characteristics desired for the expressed polypeptide. In general, expression cassettes include a promoter which is functional in the host system selected and which can be constitutive or inducible; a ribosome-binding site; an initiation codon (ATG); if necessary, a region encoding a signal peptide; a nucleotide sequence of the invention; a stop codon; and, optionally, a 3' terminal region (translation and/or transcription terminator). The open reading frame (ORF) consisting of the nucleotide sequence of the invention, alone or associated with the region encoding the signal peptide, is placed under the control of the promoter such that translation and transcription take place in the host system. The promoters and regions encoding the signal peptides are known to those skilled in the art. Among them, mention may be made in particular of the arabinose-inducible promoter (araB promoter) of *Salmonella typhimurium*, which is functional in Gram<sup>-</sup> bacteria such as *E. coli* (US 5,028,530 and Cagnon et al., Protein Engineering (1991) 4(7): 843), the promoter of the T7 bacteriophage gene encoding RNA polymerase (US 4,952,496), and the OspA and RlpB signal peptide (Takase et al., J. Bact. (1987) 169:5692).

The polypeptide expressed can be recovered in a practically purified form from the cell extract or from the supernatant, after centrifuging the recombinant cell culture. The recombinant polypeptide can, in

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particular, be purified using methods of affinity purification with the aid of antibodies, or using any other method known to those skilled in the art, for instance by genetic fusion with a small binding domain.

5           The nucleic acids of the invention can also be used in the field of vaccination, either by using a viral or bacterial host as a vehicle for releasing the DNA, or by administering the nucleic acid of interest in a free form.

10           A subject of the present invention is also (i) a vaccination vector containing a nucleic acid of the invention, placed under the control of elements allowing its expression; (ii) a pharmaceutical composition containing a therapeutically or  
15   prophylactically effective amount of said vaccination vector; (iii) a method for inducing an immune response against *Neisseria* in a vertebrate, in particular a mammal, preferably a human, said method comprising the  
20   administration to said vertebrate of an immunologically effective amount of said vaccination vector so as to cause an immune response, in particular a protective or therapeutic response to *Neisseria meningitidis*; and  
25   (iv) a method for preventing and/or treating an infection with pathogenic *Neisseria* strains, in particular with *Neisseria meningitidis*, which comprises the administration of a prophylactic or therapeutic amount of said vaccination vector of the invention to an individual requiring such a treatment.

30           In combination with the polypeptides of the invention, the vaccination vector as defined above can also comprise nucleotide sequences the expression of which allows the immune response to be stimulated, such as the sequences encoding cytokines.

35           Said vaccination vector of the invention can be administered via any route which is conventional in the field of vaccination, in particular via the parenteral route (for example subcutaneous, intradermal, intramuscular, intravenous or intraperitoneal route). The dose depends on many parameters which are known to

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those skilled in the art, such as the vector itself, the route of administration, or the weight, age or sex of the animal or of the human to be vaccinated.

A subject of the present invention is also (i)  
5 a pharmaceutical composition containing a therapeutically or prophylactically effective amount of a polynucleotide of the invention; (ii) a method for inducing an immune response against pathogenic *Neisseria* strains, in particular *Neisseria meningitidis*  
10 in a vertebrate, by administering to said vertebrate an immunologically effective amount of said polynucleotide so as to cause an immune response, in particular a protective immune response against pathogenic *Neisseria* strains, especially *Neisseria meningitidis*; and (iii) a  
15 method for preventing and for treating an infection with pathogenic *Neisseria* strains, in particular with *Neisseria meningitidis*, by administering a therapeutic or prophylactic amount of said polynucleotide to an individual requiring such a treatment.

20 The polynucleotides of the invention (DNA or RNA) can be administered to a vertebrate as they are. When a DNA molecule of the invention is used, it can be in the form of a plasmid incapable of replicating in a vertebrate cell and incapable of integrating the genome  
25 of said vertebrate. Said DNA molecule is, typically, placed under the control of a promoter suitable for expression in a vertebrate cell. Said polynucleotide used as vaccine can be formulated according to various methods known to those skilled in the art. Said  
30 polynucleotide can, in particular, be used in a naked form, free of any vehicle which promotes transfer to the target cell, such as anionic liposomes, cationic lipids, microparticles, for example gold microparticles, precipitation agents, for example  
35 calcium phosphate, or any other agent which facilitates transfection. In this case, the polynucleotide can be simply diluted in a physiologically acceptable solution, such as a sterile solution or a sterile

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buffer solution, in the presence or absence of a vehicle. When it is present, this vehicle can be preferably isotonic, hypotonic or slightly hypertonic, and has a relatively low ionic strength. It can, for example, be a sucrose solution (for example a solution containing 20% of sucrose).

Alternatively, a polynucleotide of the invention can be combined with agents which facilitate transfection. It can be, inter alia, (i) combined with a chemical agent which modifies cell permeability, such as bupivacain (WO 94/16737); (ii) encapsulated in liposomes, optionally in the presence of additional substances which facilitate transfection (WO 93/18759, WO 93/19768, WO 94/25608 and WO 95/2397, WO 93/18759 and WO 93/19768); or (iii) combined with cationic lipids, or silica, gold or tungsten microparticles.

When the polynucleotides of the invention coat microparticles, these particles can be injected via the intradermal or intraepidermal route, using the "gene gun" technique (US 4,945,050, US No. 5,015,580 and WO 94/24263).

The amount of DNA to be used as a vaccine depends, in particular, on the strength of the promoter used in the DNA construct, on the immunogenicity of the product expressed, on the individual to which this DNA is administered, on the method of administration and on the type of formulation. In general, a therapeutically or prophylactically effective amount ranging from approximately 1 µg to approximately 1 mg, preferably from approximately 10 µg to approximately 800 µg, and preferentially from approximately 25 µg to approximately 250 µg, can be administered to human adults.

The polynucleotide of the invention can be administered via any conventional route of administration, such as in particular via the parenteral route. The choice of the route of administration depends, in particular, on the formulation chosen. A polynucleotide formulated in

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combination with bupivacain is advantageously administered into muscle. When neutral or anionic liposomes, or a cationic lipid such as DOTMA (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride) or DC-Chol (3-beta-(N-(N',N'-dimethylaminomethane)carbamoyl)cholesterol) are used, the formulation can advantageously be injected via the intravenous, intramuscular, intradermal or subcutaneous route. A polynucleotide in a naked form can advantageously be administered via the intramuscular, intradermal or subcutaneous route.

The nucleotide sequences of the invention allow the construction of specific nucleotide probes and primers which can be used in diagnosis. Said probes or primers are nucleic acids having sequences identical or homologous to portions of the sequences of group I or to the sequences complementary thereto.

Preferably, said probes contain from approximately 5 to approximately 100, preferably from approximately 10 to approximately 80, nucleotides. They can contain modified bases, the sugar and phosphate residues possibly also being modified or substituted. The probes of the invention can be used in diagnostic tests, to capture or detect polynucleotides specific for pathogenic *Neisseria* strains. Such capture probes can conventionally be immobilized on a solid support directly or indirectly, by covalent bonding or by passive adsorption. A detection probe can be labelled, in particular with a radioactive isotope, an enzyme such as peroxidase or alkaline phosphatase, or enzymes capable of hydrolyzing a chromogenic, fluorogenic or luminescent substrate, or with compounds which are, themselves, chromogenic, fluorogenic or luminescent, nucleotide analogues; or biotin.

A primer generally contains from approximately 10 to approximately 40 nucleotides, and can be used to initiate enzymatic polymerization of the DNA in an amplification process (for example PCR), in an elongation process or in a reverse transcription

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method. A primer of the invention can in particular be a primer as described in Example II.1 hereinafter.

A subject of the present invention is also:

- 5 (i) a reagent containing a probe of the invention for detecting and/or identifying the presence of pathogenic *Neisseria* strains in a biological sample;
- (ii) a process for detecting and/or for identifying the presence of pathogenic *Neisseria* strains in a biological sample, said method comprising  
10 the steps consisting in a) extracting the DNA or RNA from a biological sample and denaturing it; b) exposing said DNA or said RNA to a probe of the invention, under stringent hybridization conditions, so as to detect the hybridization; and
- 15 (iii) a method for detecting and/or for identifying pathogenic *Neisseria* strains in a biological sample, in which the DNA is extracted from a biological sample and mixed together with at least one and preferably with two primers of the invention, and  
20 is amplified, for example by PCR.

As mentioned above, the polypeptides produced by the expression of the ORF sequences identified can be used as vaccination agents. The specific antigenicity of the polypeptides homologous to the  
25 polypeptides having sequences of group II can be evaluated by assaying the cross-reactivity with an antiserum directed against the polypeptides having sequences of group II. A monospecific hyperimmune antiserum can be produced against a purified  
30 polypeptide having a sequence of group II or a fusion polypeptide, for example an expression product of the MBP, GST or His-tag systems.

The specific antigenicity can be determined using various methods known to those skilled in the  
35 art, in particular the Western blot, dot blot and ELISA techniques, described below.

In the Western blot technique, the protein preparation to be tested is subjected to SDS-PAGE gel electrophoresis. After transfer onto a nitrocellulose

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membrane, the material is incubated with a monospecific hyperimmune antiserum obtained after having immunized an animal with the referent material; i.e., in the present case, with a polypeptide having an amino acid sequence of group II. This antiserum is diluted beforehand in a dilution range of approximately 1:50 to 1:5000, preferably of approximately 1:100 to 1:500. The specific antigenicity is revealed when a band corresponding to the product shows reactivity with one of the dilutions above.

In the ELISA assay, a purified protein preparation is preferably used, although a whole cell extract may also be used. Approximately 100 µl of a preparation at approximately 10 µg/ml are distributed into the wells of a plate. The plate is incubated for two hours at 37°C, and then overnight at 4°C. The plate is then washed with a phosphate buffered saline solution (PBS) comprising 0.05% of Tween 20. The wells are saturated with 250 µl of PBS containing 1% of bovine serum albumin (BSA) so as to prevent non-specific antibody binding. After incubation for one hour at 37°C, the plate is washed with the PBS/Tween buffer. The antiserum is serially diluted in PBS/Tween buffer containing 0.5% BSA. 100 µl of this dilution are added per well. The plate is incubated for 90 minutes at 37°C, washed and evaluated according to standard procedures. For example, when specific antibodies are produced in rabbits, a goat anti-rabbit peroxidase conjugate is added to the wells. The incubation is carried out for 90 minutes at 37°C and the plate is then washed. The reaction is measured by colorimetry (the reaction is positive when the optical density value is 1, if the dilution is at least 1:50, preferably at least 1:500).

In the dot blot assay, a purified protein is preferably used, it being understood that it is also possible to use a whole cell extract. Two-fold serial dilutions of a protein solution at approximately 100 µg/ml are prepared in a 50 mM Tris-HCl buffer,

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and the membrane is air-dried. The membrane is then saturated in a blocking buffer (50 mM Tris-HCl (pH: 7.5) 0.15 M NaCl, 10 g/l of skimmed milk) and incubated with a dilution of antiserum ranging from approximately 1:50 to 1:5000, preferably to approximately 1:500. The reaction is revealed in accordance with standard procedures. For example, when specific antibodies are produced in rabbits, a goat anti-rabbit peroxidase conjugate is added to the wells. The incubation is carried out for 90 minutes at 37°C.

A subject of the present invention is also (i) a pharmaceutical composition containing a therapeutically or prophylactically effective amount of a polypeptide of the invention; (ii) a method for inducing an immune response against pathogenic *Neisseria* strains in a vertebrate, by administering to said vertebrate an immunogenically effective amount of a polypeptide of the invention so as to cause an immune response, in particular a protective immune response against pathogenic *Neisseria* strains; and (iii) a method for preventing and/or for treating an infection with pathogenic *Neisseria* strains, by administering a therapeutic or prophylactic amount of a polypeptide of the invention to an individual requiring such a treatment.

The immunogenic compositions of the invention can be administered via any route which is conventional in the field of vaccination, in particular via the parenteral route (for example subcutaneous,

intradermal, intramuscular, intravenous or intra-peritoneal route). The choice of the route of administration depends on a certain number of parameters, such as the adjuvant combined with the  
5 polypeptide.

A composition of the invention contains at least one polypeptide as defined above. It can also contain at least one additional antigen of *Neisseria meningitidis* and/or *Neisseria gonorrhoeae*.

10 The polypeptides of the invention can be formulated with liposomes, preferably neutral or anionic liposomes, microspheres, ISCOMS or "virus-like" particles, in order to facilitate the transfer of the polypeptide and/or to increase the immune response.

15 The administration can be carried out with a single dose or with doses repeated, if necessary, at intervals which can be determined by those skilled in the art.

For example, an initial dose can be followed by  
20 three booster doses at intervals of one or more weeks or of one or more months. The suitable dose depends on many parameters, including the individual treated (adult or child), the specific vaccination antigen, the route of administration and the frequency of  
25 administration, the presence or absence or the type of adjuvant, and the desired effect (for example protection and/or treatment), and can be determined by those skilled in the art. If the route of  
administration is parenteral, the dose is  
30 preferentially less than 1 mg, preferably approximately 100 µg. The polypeptides and polynucleotides of the invention used as vaccination agents can be used sequentially, in a several-step immunization process. For example, a vertebrate can be initially sensitized  
35 with a vaccination vector of the invention, such as a poxvirus, for example via the parenteral route, and can then be stimulated twice with the polypeptide encoded by the vaccination vector.

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A polypeptide of the invention can also be useful as a diagnostic agent for detecting the presence of anti-*Neisseria meningitidis* and/or anti-*Neisseria gonorrhoeae* antibodies in a biological sample such as a blood sample.

A subject of the present invention is also monospecific antibodies directed against the polypeptides of the invention.

The term "monospecific antibodies" is intended to mean an antibody capable of reacting specifically with a *Neisseria* polypeptide of the invention. Such antibodies can be polyclonal or monoclonal, and can be recombinant antibodies, for example chimeric (for example consisting of a variable region of murine origin associated with a constant region of human origin), humanized and/or single-chain antibodies. Said antibodies can also be in the form of immunoglobulin fragments, for example F(ab)'2 or Fab fragments. The antibodies of the invention can be of any isotype, for example IgA or IgG, the polyclonal antibodies possibly being of a single isotype or possibly containing a mixture of several isotypes.

The antibodies of the invention directed against the polypeptides of the invention can be produced and identified using standard immunological methods, for example Western blot analysis, a dot blot assay, an ELISA assay (Coligan et al., Current Protocols in Immunology (1994) John Wiley & Sons, Inc., New York, NY). Said antibodies can be used in diagnostic processes for detecting the presence of a *Neisseria meningitidis* antigen in a sample such as, in particular, a biological sample (for example a blood sample).

The antibodies of the invention can also be used in affinity chromatography processes for purifying a polypeptide of the invention. Finally, such antibodies can also be used in prophylactic or therapeutic passive immunization methods.

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A subject of the present invention is also a diagnostic method for detecting the presence of pathogenic *Neisseria* strains in a biological sample, comprising bringing said biological sample into contact  
5 with an antibody or a polypeptide of the invention, such that an immune complex is formed, and detecting said complex which indicates pathogenic *Neisseria* strains in the organism from which the sample originates. Those skilled in the art understand that  
10 the immune complex is formed between a component of the sample and the antibody or the polypeptide of the invention, any substance not bound possibly being eliminated prior to the detection of the complex.

Thus, a reagent of polypeptide type can be used  
15 for detecting the presence of anti-*Neisseria meningitidis* and/or *Neisseria gonorrhoeae* antibodies in a sample, whereas an antibody of the invention can be used as a reagent for assaying the presence of a *Neisseria meningitidis* and/or *Neisseria gonorrhoeae*  
20 polypeptide in a sample.

For use in diagnostic applications, the reagent (for example the antibody or the polypeptide of the invention) can be in the free state or immobilized on a solid support, by direct or indirect means.

25 The direct means include passive adsorption or covalent bonding between the support and the reagent.

The term "indirect means" is intended to mean that a substance which interacts with said reagent is attached to the solid support. For example, if a  
30 reagent of polypeptide type is used, an antibody which binds to this polypeptide can be used as an anti-reagent substance, it being understood that this substance binds to an antibody which is not involved in recognizing the antibodies in the biological samples.

35 Among the indirect means which can be used, mention may also be made of the ligand receptor system, a molecule such as a vitamin possibly being grafted onto the reagent of polypeptide type, and the corresponding receptor possibly being immobilized on

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the solid phase. This is illustrated by the biotin-streptavidin system. It is also possible to add a peptide tail to the reagent, by chemical engineering or genetic engineering, and to immobilize the grafted or fused product by passive adsorption or covalent bonding with the peptide tail.

A subject of the present invention is also a process for purifying, from a biological sample, a *Neisseria* polypeptide of the invention, by affinity chromatography with a monospecific antibody of the invention. Said antibody is preferably of isotype IgG.

According to an example of implementation, a biological sample, preferably in a buffer solution, is applied to a chromatographic material, preferably equilibrated with the buffer used to dilute the biological sample, such that the polypeptide of the invention (i.e. the antigen) may adsorb to the material. The unbound components are washed and the antigen is then eluted with a suitable elution buffer, such as a glycine buffer or a buffer containing chaotropic agent, for example guanidine HCl, or a high concentration of salt (for example 3 M  $MgCl_2$ ). The eluted fractions are recovered and the presence of antigen is detected, for example by measuring the absorbance at 280 nm.

A subject of the present invention is also (i) a pharmaceutical composition containing a therapeutically or prophylactically effective amount of a monospecific antibody of the invention; and (ii) a method for preventing and/or for treating an infection with pathogenic *Neisseria* strains, by administering a therapeutic or prophylactic amount of a monospecific antibody of the invention to an individual requiring such a treatment.

To this end, the monospecific antibody of the invention is preferably of isotype IgG, and preferably fixes the complement. Said monospecific antibody according to the invention can be administered alone or in a mixture with at least one other monospecific

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antibody, specific for a different *Neisseria meningitidis* and/or *Neisseria gonorrhoeae* polypeptide, according to the invention. The amount of antibody can be determined easily by those skilled in the art. For  
5 example, a daily administration of approximately 100 to 1000 mg of antibodies over a week, or three daily doses of approximately 100 to 1000 mg of antibodies over two or three days, may be an effective dose.

The therapeutic or prophylactic effectiveness  
10 may be evaluated using standard methods known to those skilled in the art, for example by measuring the induction of an immune response or the induction of protective and/or therapeutic immunity (in newborn rats or mice), through evaluation of the bacterial load in  
15 the cerebrospinal fluid. The protection can be determined by comparing the degree of *Neisseria* infection to a control group. Protection is demonstrated when the infection is decreased in comparison with the control group. Such an evaluation  
20 can be carried out with the polynucleotides, the vaccination vectors, the polypeptides and also the antibodies according to the invention. The therapeutic or prophylactic effectiveness of a product according to the invention (polynucleotide or polypeptide) can also  
25 be evaluated in an assay for bactericidal activity, as described by Danve et al., Vaccine (1993) 11(12):1214 against the meningococcal strain of origin of the polynucleotide or polypeptide used. In the field of meningococcal vaccines, the bactericidal activity assay  
30 is, in fact, recognized as being the reference assay based upon which it is possible to make a valid prediction of the vaccination value of a product. Briefly, a product according to the invention is administered to animals such as rabbits in order to  
35 produce an antiserum against this product. Then, this antiserum is assayed for its lysis capacity. The bactericidal titre of an antiserum represents the inverse of the dilution of this antiserum for which 50% of the load of meningococci is lysed. The antiserum is

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considered to be bactericidal when the titre is higher than 4, with respect to the meningococcal strain of origin of the polynucleotide or polypeptide used. In that case, the product against which the antiserum was  
5 generated is demonstrated to be potentially advantageous from a pharmaceutical point of view.

The following examples illustrate the invention without limiting the scope thereof.

10           Legend of the figure

The attached figure represents the vector pCAMyc-His used as a cloning vector.

15           Details of the strategy for identifying the ORFs:

In order to select the ORF sequences specific for the pathogenic strains of the *Neisseria* genus, a PCR amplification is carried out on the sequences of the 118 ORFs selected after analysis with the Gene  
20 Jockey®, Codon Use®, and homology search programs. Only the sequences for which the amplification in *N. lactamica* is negative (sequences named "lactamica") are selected. In order to verify that these negative results are not "false negatives", the lactamica  
25 sequences selected are subjected to a dot blot.

A - PCR amplification:

A.1. Extraction of genomic DNAs:

30           The genomic DNAs of all of the *Neisseria* strains used in this study were prepared according to an identical protocol. The *N. meningitidis*, *N. lactamica*, *N. flava*, *N. subflava* and *N. mucosa* strains were cultured on tissues of MHA (Muller Hinton  
35 Agar, Difco) medium, and the *N. gonorrhoeae* were cultured on tissues of MHA medium supplemented with 10% of heat-treated horse blood (Biomérieux) and 1% of Isovitalex (Biomérieux). The culturing is carried out under an atmosphere containing 10%

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FOI b7D b7C b7E b7F b7G b7H b7I b7J b7K b7L b7M b7N b7O b7P b7Q b7R b7S b7T b7U b7V b7W b7X b7Y b7Z

CO<sub>2</sub>, overnight at 37°C. Then, the cells are harvested, and washed in PBS phosphate buffer (pH 7.2), and the DNA is extracted according to protocol D of the "Rapid Prep genomic DNA isolation kit for cells and tissue" (Pharmacia Biotech).

The genomic DNAs were then controlled on agarose gel for their completeness and by PCR reaction for their purity.

10            A.2. PCR reaction for screening the ORFs absent in *N. lactamica* 2314:

15            A PCR amplification was carried out on the genomic DNAs of the *N. meningitidis* strain ATCC 13090 and *N. lactamica* strain 2314 (ATCC 23970), according to the following protocol:

20            The PCR reaction was carried out on a 50 µl volume with 10 ng of genomic DNA, 250 µM of each of the dNTPs, 300 nM of each of the primers, 1X Taq DNA polymerase buffer and 2 u of Taq DNA polymerase (Appligène).

The amplification cycles are:

25	97°C	45 seconds	25 cycles
	56°C	1 minute	25 cycles
	72°C	2.30 minutes	25 cycles

30            For each of the ORFs analysed, positive and negative controls for the PCR reaction were carried out. At this stage, only the *N. meningitidis*<sup>+</sup> and *N. lactamica*- ORFs are selected.

B - Selection of the *N. meningitidis*<sup>+</sup> *N. lactamica*- ORFs by dot blot on genomic DNA:

35            The absence of a product of PCR amplification of an ORF with genomic DNA of *N. lactamica* 2314 as the matrix does not guarantee the absence of this ORF in the *N. lactamica* 2314 genome. Specifically, a certain variability in the region to which the oligonucleotides

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should hybridize may be responsible for the absence of amplified product for a given ORF.

In this context, further verification is carried out by dot blot on genomic DNA, using, as  
5 probe, the products of genomic amplification on the *N. meningitidis* strain corresponding to each of the reading frames identified. The dot blot filters contain genomic DNA of the following strains: 2 *N. lactamica* strains 8064 and 2314, one *N. flava* strain ATCC 30008,  
10 one *N. mucosa* strain ATCC 9297, 3 *N. meningitidis* serogroup B strains ATCC13090, M982 and B16B6, one *N. meningitidis* serogroup A strain Z2491, one *N. meningitidis* serogroup C strain (strain Z4182) and 2 *N. gonorrhoeae* strains MS11 and FA1090. This dot blot  
15 analysis makes it possible to validate the absence of the ORF in *N. lactamica* 2314 and 8064, and it is also an indication of the degree of variability of an ORF within the *Neisseria* strains.

The dot blot technique used is as follows.  
20 Approximately 50 ng of genomic DNA, denatured for 5 min. at 100°C, of the various *Neisseria* strains are loaded, with suction, onto a Hybond N+ nitrocellulose membrane (Amersham) placed between the jaws of a dot blot apparatus (BioRad). Then, the DNA is fixed on the  
25 membranes for 5 min with UV radiation at 315 nm.

The membranes are incubated in a prehybridization buffer (containing denatured salmon sperm DNA). They are then hybridized with a probe corresponding to the product of amplification of the  
30 ORF of interest, labelled according to a cold labelling protocol, such as the "DIG DNA labelling and detection kit" system (Boehringer Mannheim).

The ORF which does not hybridize to the genomic DNA of *N. lactamica* 2314 and 8064 is definitively  
35 selected as a potential vaccination candidate.

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Example I: Cloning

1. PCR amplification

5 Each of the ORFs was amplified by PCR using the genomic DNA of *N. meningitidis* serogroup B (strain ATCC 13090), according to standard protocol.

Two oligonucleotides, primers on the N-terminal side and on the C-terminal side were defined for each  
10 of the ORF sequences of the invention.

The primer on the N-terminal side comprises an enzyme restriction site for cloning, a CCACC Kozak sequence for translation initiation (M. Kozak, J. Mol. Biol. 196: 947-950), the ATG of the potential ORF and  
15 approximately 17 bases specific for the 5' portion of the ORF.

The primer on the C-terminal side was defined such that the ORF cloned is in fusion, in its 3' portion, with a repeat of 8 histidines and a stop codon  
20 which are present in the vector behind the multiple cloning site, hence the insertion of an "A" base in order to keep the correct reading frame after cloning and the disappearance of the stop codon of the ORF. The primer on the C-terminal side thus comprises an enzyme  
25 restriction site for cloning, an "A" base, and then approximately 20 bases specific for the 3' portion of the gene starting from the codon preceding the stop codon.

After searching for restriction sites which are  
30 absent in each of the ORFs, with the aid of the DNASTAR MapDraw subprogram (Lasergene Software), the XbaI restriction site in 5' and BglII restriction site in 3' are used for the ORF SEQ ID No. 19. For the ORF SEQ ID No. 41, the SpeI site in 5' and the BglII site in 3'  
35 are used. The XbaI restriction site in 5' and BamHI restriction site in 3' are used to clone the remaining ORFs.

The PCR mixture comprises, for a final volume of 100 µl, 10-50 ng of genomic DNA, the N-terminal and

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C-terminal primers each at 200 nM, the dNTPs each at 250  $\mu$ M, the 1X PCR buffer (composition of the 10X PCR buffer: 200 mM Tris-HCl (pH 8.8), 20 mM  $\text{MgSO}_4$ , 100 mM KCl, 100 mM  $(\text{NH}_4)_2\text{SO}_4$ , 1% TritonX-100 and 1 mg/ml of  
5 nuclease-free bovine serum albumin) and 2.5 U of polymerase.

The amplification is carried out as follows:

Step	Temperature ( $^{\circ}\text{C}$ )	Time (min.)	Number of cycles
Denaturation	97	0.45	25
Hybridization	cf. table	1	25
Elongation	72	1/kb DNA	25

10

The primers used and the PCR conditions given in the table below, in which "N. g allelic variant" means that an allelic variant is present in *Neisseria gonorrhoeae* and "N. m A allelic variant" means that an  
15 allelic variant is present in *Neisseria meningitidis* serogroup A.

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ORF No. (internal ref.)	SEQ ID No.	5' Primer	3' Primer	Polymerase	Hybridization T°
22	1-2 N.g allelic variant: 54, 55	GCT CTA GAC CAC CAT GTC TGA AGA AAA ATT GAA AAT GAG (SEQ ID n° 78)	CGG GAT CCA GAA ATG GCT GGA TTC GCT ATC AG (SEQ ID n° 79)	Tfu (Appligene)	56°C
41	3-4	GCT CTA GAC CAC CAT GAA ACA CTT ACT CAT CG (SEQ ID n° 80)	CGG GAT CCA ATA CGT AGG ACT TGG GTC (SEQ ID n° 81)	Tfu (Appligene)	43°C
42-43	5-6 N.g allelic variant: 56, 57	GCT CTA GAC CAC CAT GAA AAA ATC CCT TTT CGT TC (SEQ ID n° 82)	CGG GAT CCA TTG CGG ATA AAC ATA TTC CGC C (SEQ ID n° 83)	Tfu (Appligene)	56°C
47	7-8 N.g allelic variant: 58, 59	GCT CTA GAC CAC CAT GCG AAC GAC CCC AAC CTT C (SEQ ID n° 84)	CGG GAT CCA GAA CCG GTA GCC TAC GCC GAC (SEQ ID n° 85)	Tfu (Appligene)	56°C
55	9-10 N.g allelic variant: 60, 61	GCT CTA GAC CAC CAT GAA CAC ACG CAT CAT CGT TTC (SEQ ID n° 86)	CGG GAT CCA GCA ACG GCC TGC CGC TTT AAG (SEQ ID n° 87)	Pfu Turbo (Stratagene)	56°C
68	11-12	GCT CTA GAC CAC CAT GCT GAC GTT TAT CGG ACT G (SEQ ID n° 88)	CGG GAT CCA CGG CAG AGG CAC GAT TCC (SEQ ID n° 89)	Tfu (Appligene)	56°C

ORF No. (internal ref.)	SEQ ID No.	5' Primer	3' Primer	Polymerase	Hybridization T°
71	13-14	GCT CTA GAC CAC CAT GGG CAT CCA TCT CGA CTT C (SEQ ID n° 90)	CGG GAT CCA CAA AAG TTC CAG AAA ATC TAA CTC (SEQ ID n° 91)	Tfu (Appligene)	56°C
72	15-16 N.mA. allelic variant: 62, 63	GCT CTA GAC CAC CAT GAA TAG ACC CAA GCA ACC (SEQ ID n° 92)	CGG GAT CCA TGC CGC TTG GGG GAG GC (SEQ ID n° 93)	Pfu Turbo (Stratagene)	56°C
73	17-18 N.g allelic variant: 64, 65	GCT CTA GAC CAC CAT GAT GAA TGT CGA GGC AGA G (SEQ ID n° 94)	CGG GAT CCA CAG TTT GCC CGA CAT AC (SEQ ID n° 95)	Pfu Turbo (Stratagene)	56°C
74	19-20	GCT CTA GAC CAC CAT GAA ATT TTT TCC TGC TCC (SEQ ID n° 96)	GAA GAT CTA GAA ACT GTA ATT CAA GTT GAA G (SEQ ID n° 97)	Pfu Turbo (Stratagene)	56°C
98	21-22	GCT CTA GAC CAC CAT GAT TGA ATT TGT CCG AGC (SEQ ID n° 98)	CGG GAT CCA ACC CTG CGA CGA GTT GCG (SEQ ID n° 99)	Pfu Turbo (Stratagene)	56°C
116	23-24 N.g.allelic variant: 66, 67	GCT CTA GAC CAC CAT GCA ATA CAG CAC ACT GGC (SEQ ID n° 100)	CGG GAT CCA GTC CTT TTT CGC ACC TTG AAG (SEQ ID n° 101)	Pfu Turbo (Stratagene)	56°C
122	25-26	GCT CTA GAC CAC CAT GGA GCA GTC GGG CAA ATT C (SEQ ID n° 102)	CGG GAT CCA AGC TGT TTG GCG ATT TCG GTG (SEQ ID n° 103)	Pfu Turbo (Stratagene)	56°C

ORF No. (internal ref.)	SEQ ID No.	5' Primer	3' Primer	Polymerase	Hybridization T°
125	27-28	GCT CTA GAC CAC CAT GCA AAA CGG CGG GGG AAA G C (SEQ ID n° 104)	CGG GAT CCA GTG CCT GCG CAG CTT GGA ATC (SEQ ID n° 105)	Pfu Turbo (Stratagene)	56°C
128	29-30 N.mA. allelic variant: 68, 69	GCT CTA GAC CAC CAT GAC ATT GCT CAA TCT AAT GAT AAT G (SEQ ID n° 106)	CGG GAT CCA TTC CGC AAA TAC CTG TTT CCA ACC (SEQ ID n° 107)	Tfu (Appligene)	56°C
152	31-32 N.g allelic variant: 70, 71	GCT CTA GAC CAC CAT GAA ACA ATC CGC CCG (SEQ ID n° 108)	CGG GAT CCA TAC TTG GGC OCA ACA TGA C (SEQ ID n° 109)	Pfu Turbo (Stratagene)	56°C
153	33-34	GCT CTA GAC CAC CAT GAA TGT TTA (CGG TTT CTC (SEQ ID n° 110)	CGG GAT CCA TTT TTT AGA CGT ATT TTT AGT CG (SEQ ID n° 111)	Tfu (Appligene)	56°C
155	35-36	GCT CTA GAC CAC CAT GAT GAG TCA ACA CTC TGC C (SEQ ID n° 112)	CGG GAT CCA TCC AGT TTT TGC TCG AAG GC (SEQ ID n° 113)	Tfu (Appligene)	56°C
156	37-38	GCT CTA GAC CAC CAT GCC TTC GAG CAA AAA CTG G (SEQ ID n° 114)	CGG GAT CCA TCG TTC TTC AAT CTC CAC AAA CG (SEQ ID n° 115)	Tfu (Appligene)	56°C
157	39-40	GCT CTA GAC CAC CAT GCA CC TGG AAA G (SEQ ID n° 116)	CGG GAT CCA TTC AAT TCG CTT CAA CAA TG (SEQ ID n° 117)	Tfu (Appligene)	56°C



ORF No. (internal ref.)	SEQ ID No.	5' Primer	3' Primer	Polymerase	Hybridization T°
158	41-42 N.mA. allelic variant: 72, 73	GGA CTA GTC CAC CAT GGC TTC CAA CCA ACG TTA CCG (SEQ ID n° 118)	GAA GAT CTA AGC CGC GTT CCC TTC CAA AAA ATC (SEQ ID n° 119)	Tfu (Appligene)	56°C
159	43-44 N.mA. allelic variant: 74, 75	GCT CTA GAC CAC CAT GCC GCA AAT TAA AAT TCC C (SEQ ID n° 120)	CGG GAT CCA AAA ACA ATC TTC CGG CAC CC (SEQ ID n° 121)	Tfu (Appligene)	56°C
161	45-46	GCT CTA GAC CAC CAT GCC CAC GCC GTT TTG TTG (SEQ ID n° 122)	CGG GAT CCA TTG GGC AAC GAC GAA GGC AC (SEQ ID n° 123)	Tfu (Appligene)	56°C
163-1	47-48	GCT CTA GAC CAC CAT GAG AAT AGA GAT CAC ACC (SEQ ID n° 124)	CGG GAT CCA TGG CTC AAT CCT TTC TGC (SEQ ID n° 125)	Pfu Turbo (Stratagene)	56°C
163-2	49-50	GCT CTA GAC CAC CAT GAT TCA CGT TTC GGC AGT G (SEQ ID n° 126)	CGG GAT CCA ACC TGC TTC ATG GGT GAT TC (SEQ ID n° 127)	Tfu (Appligene)	56°C
167-168	51-52 N.gallelic variant: 76, 77	GCT CTA GAC CAC CAT GAA TTC GAC CGC AAG TAA AAC (SEQ ID n° 128)	CGG GAT CCA AAT CCC TCT GCC GTA TTT G (SEQ ID n° 129)	Tfu (Appligene)	56°C

2- Cloning, transformation and selection of recombinants

The cloning vector used is the 6.357 kb vector pCA/Myc-His or pM1070 (cf. figure), derived from the plasmid pCDNA 3.1 (Invitrogen). pCA/Myc-His comprises, in particular, the CMV iel promoter (bases 249-902), intron A of the CMV iel gene (Chapman et al., 1991 Nucleic Acids Research, 19, 3979-3986), a multiple cloning site (bases 1792-1852) with the PmlI, EcoRV, NotI, XbaI, BamHI, KpnI and HindIII sites, a sequence encoding a polyhistidine and a stop codon (bases 1908-1928), a bgh 3' termination sequence (bases 1853-2197) and the ampicillin resistance gene for selecting the recombinant clones in *E. coli*.

After purification (GeneClean Bio101 kit), the PCR amplification products are digested for 2 hours at 37°C with the appropriate enzymes (XbaI-BamHI, XbaI-BglII or SpeI-BglII), in a final reaction volume of 20 µl. The digestion products are then ligated with the vector pCA/Myc-His, digested beforehand with XbaI and BamHI, according to the "Rapid DNA Ligation Kit" protocol (Boehringer Mannheim). 15 µl of the ligation is used to transform 100 µl of competent *E. coli* XLI-blue cells (Novagen). The cells are incubated for 30 minutes in ice, 30 seconds at 42°C and 2 minutes in ice. Then, 500 µl of LB medium without antibiotics are added, and the mixture is incubated for 1 hour at 37°C. Next, 50 and 550 µl of the culture are plated out on plates containing LB medium plus ampicillin (50 µg/ml final concentration), and incubated overnight at 37°C.

The following day, 36 colonies are placed in culture in 2 ml of LB plus ampicillin (50 µg/ml) and incubated overnight at 37°C.

The following day, the plasmid DNA is extracted according to the Qiagen mini-prep protocol (Qiagen) and the recombinants are identified by enzymatic restriction followed by agarose gel electrophoresis. The cloning junctions are then verified by sequencing.

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Example II: Evaluation of the protective activity of the ORFs of the invention

5 A. Preparation of the DNA intended for the immunization experiments:

10 An isolated colony of a recombinant clone is used to inoculate a preculture in LB medium + ampicillin, and 5 ml of this preculture represents the inoculum of a 2.5 litre culture in LB medium + ampicillin. The purification protocol for preparing the plasmid DNA is that described in the EndoFree Giga Kit (Qiagen). The purified DNA is eluted from the purification column with a 10 mM Tris-HCl, 1 mM EDTA  
15 buffer, pH 8, and stored at -20°C. Before injection, the purified recombinant plasmid is diluted to 100 µg/ml with water (of injectable preparation quality) and the NaCl concentration is brought to 150 mM.

20 B. Production of a specific polyclonal serum:

25 B.1. Hyperimmunization in an animal model:

The animal model used is the mouse or the rabbit. The route of administration of the injected DNA is the intramuscular or intradermal route. The recombinant plasmids to be injected are optionally applied to beads if they are injected into animals  
30 using a gene gun apparatus (BioRad). The immunization protocol follows a scheme comprising two injections, 3 weeks apart.

35 B.2. Analysis of the bactericidal activity of the antibodies induced:

Ten days after the final injection, the animals are bled and the sera are analysed using the bactericidal activity assay according to the protocol of Danve et al., Vaccine (1993) 11 (12):1214. Briefly,

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the sera are incubated at various dilutions (2-fold) in the presence of rabbit complement and of meningococci cultured in the presence or absence of an iron-chelating agent. The bactericidal titre of a serum represents the inverse of the dilution of this antiserum for which 50% of the bacteria are lysed.

It is considered that the antiserum is not bactericidal when its titre is lower than 4 against the homologous strain.

When the bactericidal titre corresponds to a 4-fold seroconversion against the homologous strain, the bactericidal activity of the antiserum is analysed against other *Neisseria* strains in order to measure the extent of the cross-reactivity of the antiserum of interest.

### Example III: Production of purified recombinant proteins

#### 1. Recombinant production of proteins

##### a. Preparation of transformants:

The PCR product obtained is then digested at 37°C for two hours with restriction enzymes, in 20 µl of reaction volume. The digestion product is ligated into a plasmid pET28a (Novagen) which is cleaved in a similar way and which is dephosphorylated, before ligation, by treating with calf intestine alkaline phosphatase. The fusion gene constructed in this way allows the one-step affinity purification of the resulting fusion protein, due to the presence of histidine residues at the N-terminal end of the fusion protein, which are encoded by this vector.

The ligation reaction (20 µl) is carried out at 14°C overnight, before transforming 100 µl of fresh competent *E. coli* XL1-blue cells (Novagen). The cells are incubated on ice for two hours, and then subjected to a heat shock at 42°C for 30 seconds, before being returned to the ice for 90 seconds. The samples are

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supernatant is filtered through a 0.45  $\mu$ m membrane. The supernatant is loaded onto an IMAC column (12 ml of resin) which has been prepared by immobilizing Ni<sup>++</sup> cations according to the manufacturer's recommendations (Pharmacia). The column is washed with 10 column volumes of 20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 60 mM imidazole. The recombinant protein is eluted with six volumes of 20 mM Tris-HCl (pH: 7.9), 0.5 M NaCl, 500 mM imidazole, 0.1% Zwittergent 3-14.

The elution profile is controlled by measuring the absorbance of the fractions at an optical density of 280 nm. An aliquot fraction is analysed on an SDS-PAGE gel and stained with Coomassie blue (Phast System - Pharmacia), and the fractions corresponding to the protein peak are then pooled and concentrated. In order to eliminate the elution buffer, the fraction is passed over a G24 Sephadex column (Pharmacia) and equilibrated in PBS buffer (pH: 7.4). The protein solution is sterilized by filtration through a 0.45  $\mu$ m membrane, and the protein concentration is determined using the BCA micromethod (Pierce). The protein solution is stored at -70°C.

**Example IV: Production of monospecific polyclonal antibodies**

**1. Rabbit hyperimmune antiserum**

100  $\mu$ g (in total) of the polypeptide purified in Example III, in the presence of complete Freund's adjuvant in a total volume of approximately 2 ml, are injected into New Zealand rabbits, both subcutaneously and intravenously. 21 and 42 days after the initial injection, the booster doses, which are identical to the initial doses, are administered in the same way, with the exception that incomplete Freund's adjuvant is used. 15 days after the final injection, the animal's serum is recovered, decomplexed and filtered through a 0.45  $\mu$ m membrane.

## 2. Mouse hyperimmune ascites fluid

10-50 µg of the purified fusion polypeptide obtained in Example II, in the presence of complete Freund's adjuvant, in a volume of approximately 200 µl, are injected subcutaneously into 10 mice. 7 and 14 days after the initial injection, booster doses, which are identical to the initial doses, are administered in the same way, with the exception that incomplete Freund's adjuvant is used. 21 and 28 days after the initial injection, the mice receive 50 µg of the antigen alone, intraperitoneally. On the 21st day, the mice are also injected intraperitoneally with 180/TG CM26684 sarcoma cells (Lennette & Schmidt, Diagnostic procedures for viral, rickettsial, and chlamydial infections, (1979) 5th Ed. Washington DC, American Public Health Association). The ascites fluids are harvested 10 to 13 days after the first injection.

## Example V: Purification of the polypeptides of the invention by immunoaffinity

### 1. Purification of specific IgG

An immune serum as prepared in Example IV is applied to a Fast Flow Sepharose 4 protein A column (Pharmacia) equilibrated with 100 mM Tris-HCl (pH: 8.0). The resin is washed by applying 10 column volumes of 100 mM Tris-HCl and 10 volumes of 10 mM Tris-HCl (pH: 8.0) to the column. The IgGs are eluted with a 0.1 M glycine buffer (pH: 3.0) and are collected by 5 ml fraction, to which 0.25 ml of 1 M Tris-HCl (pH: 8.0) are added. The optical density of the eluate is measured at 280 nm and the fractions containing the IgGs are pooled and, if necessary, stored at -70°C.

### 2. Column preparation

A suitable amount of CNBr-activated Sepharose 4B gel (1 g of dried gel providing approximately 3.5 ml of hydrated gel, and the capacity of the gel ranging from 5 to 10 mg of coupled IgG per ml of gel)

manufactured by Pharmacia (17-0430-01) is suspended in 1 mM HCl buffer and washed, using a Buchner funnel, by adding small amounts of 1 mM HCl buffer. The total volume of the buffer is 200 ml per gram of gel.

5           The purified IgGs are dialysed for four hours at  $20 \pm 5^\circ\text{C}$  against 5 volumes of 500 mM PBS buffer (pH: 7.5). Then, they are diluted in 500 mM of PBS (pH: 7.5) for a final concentration of 3 mg/ml.

10           The IgGs are incubated with the gel overnight at  $5 \pm 3^\circ\text{C}$ , with stirring. The gel is packed into a chromatography column and washed with 2 column volumes of 500 mM phosphate buffer (pH: 7.5) and then one volume of 50 mM NaCl sodium buffer (pH: 7.5). The gel is then transferred to a tube, then incubated with  
15 100 mM of ethanolamine (pH: 7.5) for 4 hours at room temperature with stirring, and then washed twice with two column volumes of PBS. The gel is then stored in PBS merthiolate at 1/10 000. The amount of IgG coupled to the gel is determined by measuring the optical  
20 density at 280 nm of the IgG solution and of the direct eluate.

### 3.           Adsorption and elution of the antigen

25           A solution of antigen in 50 mM Tris-HCl (pH: 8.0), 2 mM EDTA, for example the supernatant obtained in Example III.2 after treatment with Benzonase, centrifugation and filtration through a 0.45  $\mu\text{m}$  membrane, is applied to a column equilibrated with 50 mM Tris-HCl (pH: 8.0), 2 mM EDTA, at a flow  
30 rate of approximately 10 ml/hour. Then, the column is washed with 20 volumes of 50 mM Tris-HCl (pH: 8.0), 2 mM EDTA. Alternatively, batch adsorption can be carried out, in which the mixture is left overnight at  $5 \pm 3^\circ\text{C}$ , with stirring.

35           The gel is washed with 2 to 6 volumes of 10 mM PBS buffer (pH: 6.8). The antigen is eluted with a 100 mM glycine buffer (pH: 2.5). The eluate is collected in 3 ml fractions, to which 150  $\mu\text{l}$  of 1 mM PBS buffer (pH: 8.0) are added. The optical density is

0930433-031601



measured at 280 nm for each fraction; those containing the antigen are recovered and stored at -20°C.

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Fragments of the genome of *N. meningitidis* Z2491  
described in patent application WO 98/02547

(2) INFORMATION FOR SEQ ID NO: 70A:

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- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 243 base pairs
  - (B) TYPE: nucleotide
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

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- (ii) MOLECULE TYPE: DNA (genomic)

- (iii) HYPOTHETICAL: NO

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- (iv) ANTISENS: NO

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 70A:

GATCAGACCC ATTTTCAGCG CACCGTAAGC GCGGATTTTC TCGAATTTT CCAAAGCTGC	60
GGCATCGTTG TTGATGTCGT CTGCAACTC TTTGCCCGTG TAGCCCAAGT CGGCGGCATT	120
CAGGAAAACG GTCGGAATGC CCGCGTTGAT GAGCGTGGCT TTCAAACGGC CTATATTCGG	180
CACATCAATT TCATCGACCA AATTGCCGGT TGGGAACATA CTGCCTTCGC CGTCGGCTGG	240
ATC	243

20

(2) INFORMATION FOR SEQ ID NO: 73A:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 120 base pairs
  - (B) TYPE: nucleotide
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

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- (ii) MOLECULE TYPE: DNA (genomic)

- (iii) HYPOTHETICAL: NO

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(iv) ANTISENS: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 73A:

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CGGTCAGAAA CAGGCAAGGT AATGAAAATG CCTGAGGCAC GGACTGTGCT GCGAACGAAA 60  
ACTCCTTACC GAAGTCTTCT ATACCCAGGC TCAATAGCCG CTCAAGGAGA GAGCTATCAT 120

(2) INFORMATION FOR SEQ ID NO: 74A:

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(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 120 base pairs  
(B) TYPE: nucleotide  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA (genomic)  
(iii) HYPOTHETICAL: NO

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(iv) ANTISENS: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 74A:

CGGTCAGAAA CAGGCAAGGT AATGAAAATG CCTGAGGCAC GGACTGTGCT GCGAACGAAA 60  
ACTCCTTACC GAAGTCTTCT ATACCCAGGC TCAATAGCCG CTCAAGGAGA GAGCTATCAT 120

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(2) INFORMATION FOR SEQ ID NO: 77A

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 269 base pairs  
(B) TYPE: nucleotide  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA (genomic)

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09030433-081601

(iii) HYPOTHETICAL: NO

(iv) ANTISENS: NO

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 77A:

```
CGGAGCATAA AATCGTTATT AAAGATAATG GTATAGGAAC GAGCTTCGAT GAAATCAATG      60
ATTTTTATTT GAGAATCGGT CGGAACAGAA GGGAAGAAAA ACAAGCCTCC CCGTGCGGAA      120
GAATCCAAC GGGTAAAAAA GGCCTTGTA AATTGGCATT ATTCGGGCTT GGCAACAAAA      180
TTGAAATTC TACTATCCAG GGAAACGAAA GGGTTACTTT TACTTTGGAT TATGCAGAGA      240
TTCGAAGAAG CAAGGGTATT TATCAACCG      269
```

(2) INFORMATION FOR SEQ ID NO: 80A:

10

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 207 base pairs
  - (B) TYPE: nucleotide
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

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(iv) ANTISENS: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 80A:

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CGGGTCGCTT TATTTTGTGC AGGCATTATT TTTCATTTTT GGCTTCACAG TTTGAAATA      60
TTGTGTATCG GGGGGGGGTA TTGCTGACG TAAAAACTA TAAACGCCGC GCAAAATATG      120
GCTGACTATA TTATTGACTT TGATTTTGTG CTGCGCGGTG ATGGATAAAA TCGCCAGCGA      180
TAAAGAATTT GCGAGAACCT GATGCCG      207
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(2) INFORMATION FOR SEQ ID NO: 81A:

09830437-081601

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(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

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(iii) HYPOTHETICAL: NO

(iv) ANTISENS: NO

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120

18C

224

(2) INFORMATION FOR SEQ ID NO: 87A:

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(A) LENGTH: 273 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

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(iv) ANTISENS: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 87A:

AATTTCCACC TATGCCCTAC GCAGCGATTA TCCGTGGTTT ACCCAAAGGG TGATTATGGC 60  
AAAAGCGCGG GGTGAGCGA CCGCCTTTTG TTGCCGGCGT TCAAACGGGT TTTGATAGGA 120  
AATGCAGGCA CGAAGCCTCG GCTGATTGTG ATGCACCTGA TGGGTTCGCA CAGTGATTTT 180  
TGCACACGTT TGGATAAGGA TCGCGGGCGG TTTCAGTATC AACTGAAAA AATATCCTGC 240  
TATGTTTCCA TCAATCGCGC AAACCGATAA ATT 273

(2) INFORMATION FOR SEQ ID NO: 88A:

5

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 270 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

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(iv) ANTISENS: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 88A:

AATTCCTCCG CACGGGGAGG CTGTGTTTTT TCCCTTCTG TTCCGACCGA TTCTCAAATA 60  
AAAATCATTG ATTTCAICGA AGTTCATTCC TATACCATTA TCTTTAATAA CGATTTTATG 120  
CTCCGGTTTA TCGAATAACC TAACTTCCAC TTCCGTAGCA CATGCATCGT AGGCATTGCG 180  
TATCAACTCG GCAATCGCAG GAACAGTGTG CGAATACAAT CTTTACACCC AAATGTTTGA 240  
TTACGGTTGG CTCGAAACTC AATTCAATT 273

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(2) INFORMATION FOR SEQ ID NO: 89A:

(i) SEQUENCE CHARACTERISTICS:

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(A) LENGTH: 267 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

09030433-081601

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

5 (iii) HYPOTHETICAL: NO

(iv) ANTISENS: NO

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 89A:

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AATTATGAAC ACACGCATCA TCGTTTCGGC TGCCTTCGTT GCGTTGGCAT TAGCAGGTTG      50
CGGCTCAATC AATAATGTAA CCGTTTCCGA CCAGAAACTT CAGGAACGTG CCGCGTTTGC      100
CTTGGGCGTC ACCAATGCCG TAAAAATCAG CAACCGCAGC AATGAAGGCA TACGCATCAA      150
CTTTACCGCA ACTGTGGGTA AGCGCGTGAC CAATGCTATG TTACCAGTGT AATCAGCACA      200
ATCGGCGTTA CCACTTCCGA TGCAATT                                           267
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(2) INFORMATION FOR SEQ ID NO: 94A:

15 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 308 base pairs  
(B) TYPE: nucleotide  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

25 (iv) ANTISENS: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 94A:

09630433-081601





(B) TYPE: nucleotide  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTISENS: NO

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 98A:

AATTTGTCGG CAATCTTCCC GGGTCGCTTT ATTTTGTGCA GGCATTATTT TTCATTTTTG	60
GCTTGACAGT TTGGAGATAT TGTGTATCGG GGGGGGGTAT TTGCTGACGT AAAAAACTAT	120
AAACGCCGCA GCAAAATATG GCTGACTATA TTATTGACTT TGATTTTGTC CTGCGCGGTG	180
ATGGATAAAA TCGCCAGCGA TAAAGATTTC CGAGAACCTG ATGCCGCCCT GTTGTGAAT	240
ATTTTCGACC TGTAATTACG ATTGGGCTTC CGCGCCGGCA CAATATGCCG CCAAGCGGCG	300
CCCACATTTT GGAAGC	316

09330433-081501